Cell Differentiation and Development, 30 (1990) 109–116
Elsevier Scientific Publishers Ireland, Ltd.

CELDIF 00658

A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells

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(Accepted 5 January 1990)

A cell line, ATDC5, isolated from a differentiating culture of AT805 teratocarcinoma expressed a fibroblastic cell phenotype in a growing phase. With the addition of 10 μg/ml insulin to the medium, cells continued to grow even in a postconfluent phase, formed cartilage nodule-like cell aggregates, were stained with Alcian blue and produced cartilage-specific proteoglycan and type II collagen, typical marker molecules for chondrogenesis. Since ATDC5 cells also differentiated into unidentifiable pigmented cells, they are apparently composed of undetermined cells. ATDC5, therefore, provides a good model system with which to understand chondrogenic differentiation.

Chondrogenic cell; Teratocarcinoma; Insulin; Cartilage-specific proteoglycan; Collagen type II; Pigmented cell

Introduction

Chondrocytes have been widely used in studies on the process of cell differentiation and various factors and conditions shown to be necessary for chondrogenic differentiation, such as cellular interaction, tissue interaction, NAD, bone morphogenic protein, and c-AMP (Hunter and Caplan, 1983). Neither the cellular mechanisms by which those factors or conditions affect the differentiation nor the nature of chondrogenic precursor cells has yet been clarified, however.

Isolation of a chondrogenic cell line may be quite useful in describing the mechanism of chondrogenic differentiation. Establishment of such a chondrogenic precursor cell line is not easy, because it is difficult to maintain chondrogenic cell phenotypes for a long time in culture and it is also difficult to establish a cell line from chick embryonic cells, which are usually used as sources for studying chondrogenesis.

Mouse teratocarcinoma stem cells are believed to be derived from and equivalent to embryonic ectoderm cells of the egg-cylinder stage embryo, which were shown to differentiate into all types of somatic cells and germ cells of a mouse (Martin, 1975). Various teratocarcinoma stem cell lines have been established; some of them can be maintained as a growing population of undifferentiated cells or can be induced to differentiate. They usually

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differentiate into chondrocytes after their injection into a syngeneic host (Martin, 1975). However, they rarely differentiate into chondrocytes in vitro except one system that we reported previously (Atsumi et al., 1985). In it, PCC3/A/1 teratocarcinoma cells constantly differentiated into chondrocytes in vitro after clonal growth for several days in serum-free 3F medium (see Materials and Methods) with a feeder layer of mitomycin-C-treated KCF cells, which were unidentifiable fibroblastic cells derived from another teratocarcinoma cell line OKTC3H-1 (Noguchi et al., 1982) and chondrogenic differentiation was always preceded by fibroblastic cell differentiation.

As a teratocarcinoma stem cell is at a very early differentiation stage to be able to yield various types of somatic cells, it is improbable that chondrocytes are directly differentiated from the stem cell. There may be some intermediate differentiation stages between teratocarcinoma stem cells and chondrocytes. In normal development, chondrocytes are differentiated from mesodermal cells or neural crest cells; both cells usually express fibroblastic phenotypes in vitro. We therefore speculated that some fibroblastic cells derived from teratocarcinoma stem cells might be the precursor cells of chondrocytes (Atsumi et al., 1985). Indeed, two fibroblastic cell lines were isolated from mouse teratocarcinomas which differentiate into chondrocytes when injected into a syngeneic mouse (Morgan et al., 1983; Nicolas et al., 1980). However, neither cell has been reported to differentiate into chondrocytes in vitro. Two established cell lines, C3H10T1/2 (Taylor and Jones, 1979) and RJC3.1 (Grigoriadis et al., 1988), have been reported to be chondrogenic, but their chondrogenic differentiation appears to take place at a low frequency. In the present paper, our success in isolating a chondrogenic cell line with a high differentiating frequency from mouse teratocarcinoma-derived fibroblastic cells is described.

Materials and Methods

Cells and cell culture

The cell line, AT805, was described previously (Takeichi et al., 1981). It is a feeder-independent teratocarcinoma stem cell line, established from the embryoid body line SSEB (a subline of SEBIII (Amano et al., 1978)) originally derived from OTT6050 (Stevens, 1970). All stock cultures were grown in 3F medium which is a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (F12) containing 10 μg/ml human transferrin (Sigma), 10 μg/ml bovine insulin (Sigma), and 3×10^{-8} M sodium selenite (Darmon et al., 1981) supplemented with 5% fetal calf serum (FCS). DF medium was a 1 : 1 mixture of DMEM and F12 containing 3×10^{-8} M sodium selenite.

Antisera and immunostaining

Production and characterization of anti-cartilage-specific proteoglycan (CSPG) rabbit serum were described by Kimata et al. (1981). Anti-type II collagen rabbit serum was obtained from Advance (Tokyo). Normal swine serum, swine anti-rabbit Ig, peroxidase-conjugated rabbit anti-peroxidase and 3-amino-acetyl carbazole were obtained from Dakopatts (Denmark). Cells in a Lab Tec chamber well (Miles) were washed with HCMF (Hepes-buffered Ca^{2+}- and Mg^{2+}-free saline) (Takeichi et al., 1981) and then fixed with 100% methanol at $-20\,^{\circ}C$ for 2 min. Cells were treated with normal swine serum, anti-CSPG or anti-type II collagen rabbit serum, anti-rabbit Ig swine serum and finally horseradish-peroxidase-conjugated anti-peroxidase rabbit Ig complex. The peroxidase-catalyzed reaction was carried out in 1 mM 3-amino-acetyl carbazole/0.01% H_2O_2/0.1 M sodium acetate buffer (pH 5.2) at 37 °C for 20 min.

Histochemical staining with Alcian blue

Cells were fixed with 100% methanol at $-20\,^{\circ}C$ for 2 min. They were incubated with 0.1% Alcian blue (Sigma) in 0.1 N HCl for 2 h at 25 °C (Lev and Spiecer, 1964), then they were washed three times with distilled water and observed with a microscope.

Quantitation of chondrogenesis by Alcian blue staining

The extent of chondrogenic differentiation was estimated by measuring the amount of extractable dye. Stained cultures in a 6-cm Petri dish were

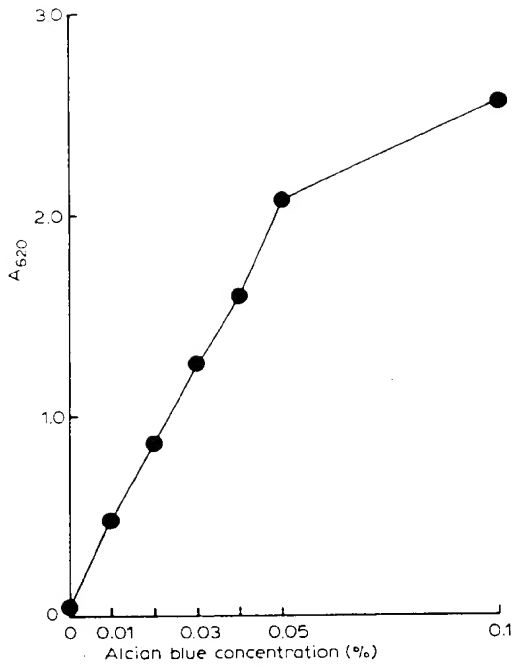

Fig. 1. Quantitative relationship between the concentration of Alcian blue and A_{620}. For preparing the indicated concentrations of Alcian blue solution, 0.1% Alcian blue in 0.1 N HCl was diluted in series with 6 M guanidine-HCl.

extracted with 2 ml of 6 M guanidine-HCl for 8 h at 25 °C. Total optical density of extracted dye in 6 M guanidine-HCl at 620 nm was measured and designated as A_{620} (Matsutani and Kuroda, 1978; San Antonio and Tuan, 1986). The linear correlation between A_{620} and the concentration of Alcian blue was obtained when the A_{620} was lower than 2.0 (Fig. 1). Therefore, it is possible that A_{620} is used as an index representing the amount of Alcian blue, with the limitation that it should be lower than 2.0.

Results

Isolation of chondrogenic cell line ATDC5

Murine teratocarcinoma AT805 cells can differentiate into a wide range of somatic cells including chondrocytes after their injection into a syngeneic host. They differentiated into chondrocytes in vitro hardly at all, but, spontaneously, they differentiated into fibroblastic cells. In order to characterize these cells, we subjected them to clonal analysis. Into a 6-cm plastic Petri dish, 10^3 cells were inoculated; 2 to 5 colonies were formed in each dish. A few colonies contained a round cell cluster in the center which morphologically resembled chondrocytes derived from the PCC3/A/1 teratocarcinoma (Atsumi et al., 1985). As one such colony was stained with Alcian blue, other colonies containing these round cell clusters were cloned and after three repeats of the cloning, one clone was established and named ATDC5.

ATDC5 exhibited a fibroblastic cell morphology at the subconfluent stage (Fig. 2A). The cells did not stop growing after confluence and the formed cell aggregates looked like cartilage nodules if the medium was changed every day. Cells in the aggregates exhibited a round morphology (Fig. 2B). These cartilage nodule-like aggregates were intensely stained with Alcian blue (Fig. 2C), and also stained immunohistochemically with anti-CSPG (Figs. 2D and 2E) and anti-type II collagen (Fig. 2F) antibodies.

High dose insulin-dependent accumulation of Alcian blue-positive matrix

We first used 3F medium supplemented with 5% FCS both to maintain and to induce differentiation of ATDC5. To investigate whether each component of the culture medium is necessary for the differentiation, cells were cultured in various media with different compositions (Table I). All media were commonly supplemented with 5% FCS. Into a 6-cm Petri dish, 5×10^5 cells, suspended in

TABLE I

The chondrogenic differentiation of ATDC5 cells in various culture media

	Degree of differentiation (A_{620})	
	Without insulin	With insulin [a]
DF	0.059	1.239
DF + Transferrin [b]	0.058	1.156
DMEM	0.063	0.482
F12	0.068	0.625

The A_{620} value of 6 M guanidine-HCl was 0.049 (background). Each medium was commonly supplemented with 5% FCS. Each value is the mean of triplicate data.
[a] 10 μg/ml; [b] 10 μg/ml.

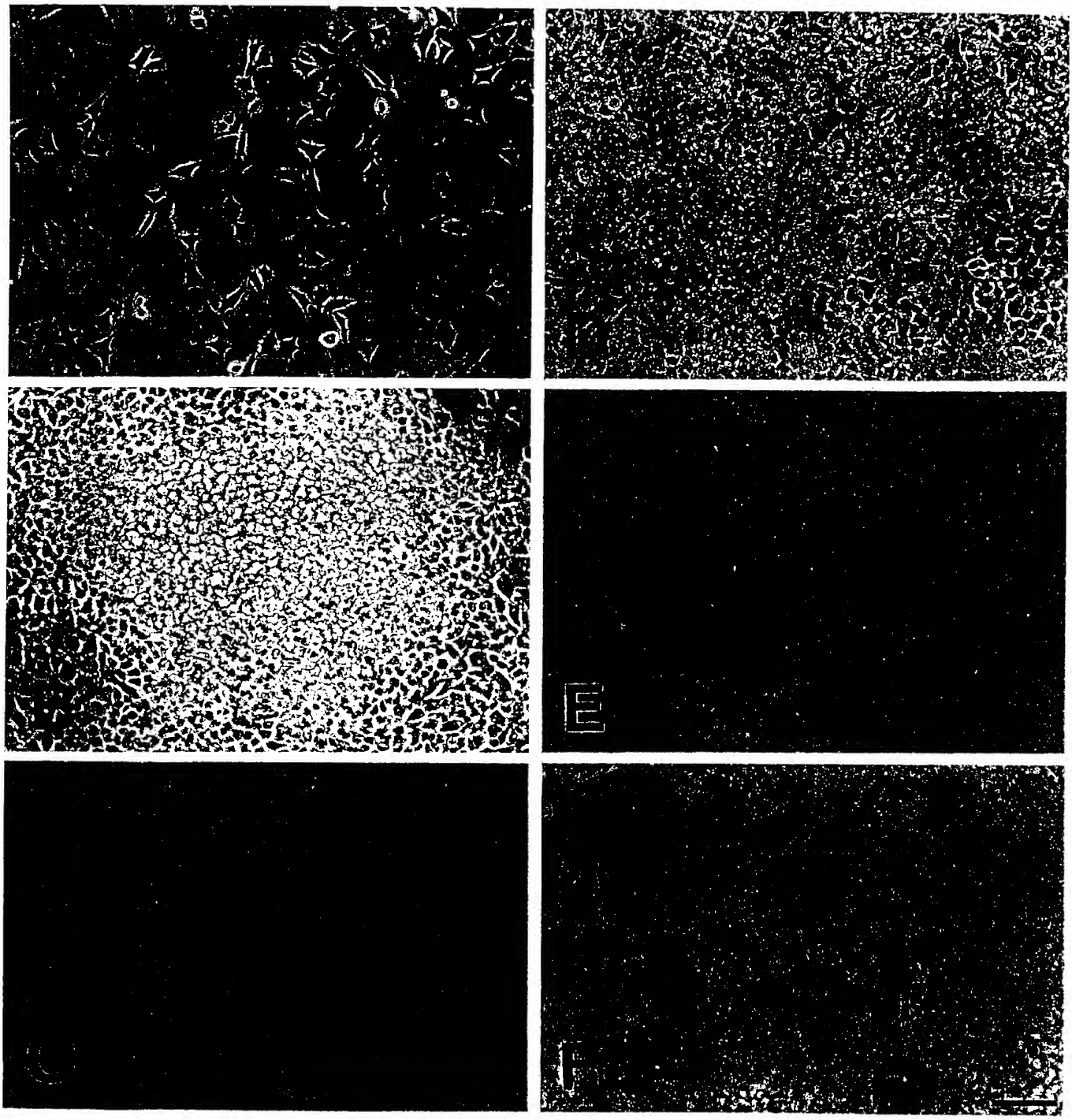

Fig. 2. (A) Morphology of ATDC5 in growing phase; (B) morphology of a cartilage nodule-like aggregate of ATDC5; (C) photomicrograph of differentiated ATDC5 cells stained with Alcian blue; (D) phase-contrast photomicrograph of differentiated ATDC5 cells stained with anti-CSPG antibody; (E) the same field as (D) without phase-contrast; (F) photomicrograph of differentiated ATDC5 cells stained with anti-type II collagen. Bar, 50 μm.

5 ml of each medium, were inoculated. The medium was changed daily for 14 days at which time the cells were fixed and stained with Alcian blue. The amount of extractable dye bound was measured and used as a parameter of chondrogenic differentiation; the results are summarized in Table I. In each medium without the addition of 10 μg/ml insulin, only a few small clumps of cells were stained and very little dye was bound. The addition of transferrin to the medium had no effect at all, while the addition of insulin greatly promoted the differentiation. In DMEM of F12, to which insulin was added, ATDC5 cells also differentiated, but the differentiation was not as remarkable as in the DF medium.

Effects of various concentrations of insulin on ATDC5 differentiation

The above results showed that insulin was necessary for the differentiation of ATDC5. Next we examined the effects of various concentrations of insulin on this differentiation. One hundred thousand ATDC5 cells in DF medium supplemented with 5% FCS and various concentrations of insulin were inoculated into 6-cm Petri dishes. After 14 days the cells were fixed and stained with Alcian blue followed by the measurement of extractable dye (Fig. 3). When at least 0.1 μg/ml insulin was added, a significant promotion of differentiation was observed. The highest incidence of differentiation was observed with the addition of 10 to 50 μg/ml insulin. Differentiation was inhibited by 100 μg/ml insulin.

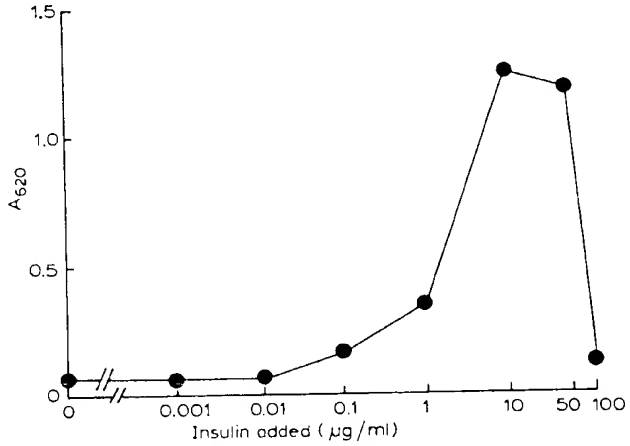

Fig. 3. Effects of various concentrations of insulin added to the medium on the chondrogenic differentiation of ATDC5 cells. Cells were cultured for 14 days in DF medium supplemented with 5% FCS and the indicated concentration of insulin, and then stained with Alcian blue. The bound dye was extracted with 6 M guanidine-HCl. The concentration of extracted dye was measured at 620 nm and designated A_{620}.

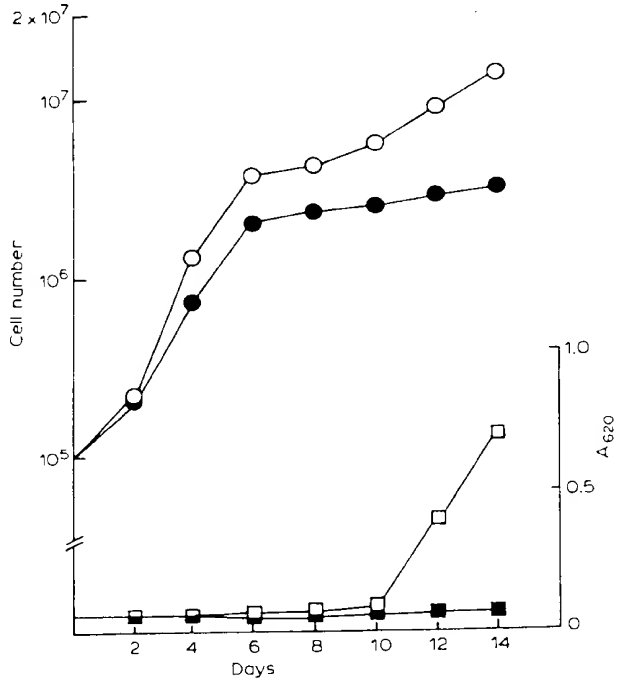

Fig. 4. (A) Growth curves and (B) the time course of the stainability with Alcian blue of ATDC5 in medium with or without the addition of 10 μg/ml insulin. (●) cell number in DF medium supplemented with 5% FCS and 10 μg/ml insulin; (●) cell number in DF medium supplemented with 5% FCS alone; (□) A_{620} of ATDC5 cells grown in medium supplemented with 5% FCS and 10 μg/ml insulin; (■) A_{620} of ATDC5 cells grown in DF medium supplemented with 5% FCS alone.

Growth curves of ATDC5

Thus insulin enhanced not only the accumulation of the Alcian blue staining materials but also the cell proliferation in a concentration-dependen manner. To examine its effect on the growth o ATDC5, 10^5 cells were inoculated into a 6-cm Petri dish suspended in 5 ml of each medium, DF with 5% FCS or DF with 5% FCS and insulin (Fig. 4). The medium was changed every day. In DF with 5% FCS, cells became confluent at th

density of 2×10^6 cells per 6-cm dish and stopped proliferating. In DF with 5% FCS and insulin, on the other hand, cells reached confluence when they increased up to 4×10^6 per dish. Two or three days after the confluence the cells began to proliferate again and to form cartilage nodule-like cell aggregates. Doubling time before the confluence was 14.4 h, whereas that afterwards was 48 h. It should be noted that cells began to be stained with Alcian blue several days after they became confluent (Fig. 4). The production of Alcian blue staining materials appeared to be correlated with the insulin-dependent cell reproliferation.

Expression of cartilage specific-proteoglycan

To determine whether or not the accumulation of the Alcian blue staining materials was due to the synthesis of cartilage-specific proteoglycan (CSPG), cells were cultured in Lab Tec chamber plates and immunostained. In DF medium supplemented with 5% FCS and insulin, cells in a growing phase were not stained with anti-CSPG antibodies. Just after entering a confluent phase, the cells had not yet formed cell aggregates; only a few were stained with the antibodies (Fig. 5A and B). When culturing was continued, and cartilage nodule-like aggregates were formed, most cells in the aggregates became intensely stained with anti-CSPG antibodies. In DF medium supplemented only with 5% FCS, ATDC5 cells did not form the cell aggregates even in the confluent phase; however, a few were stained with the antibodies (data not shown).

Pigmented cell differentiation

Under the same conditions as in the chondrogenic differentiation, ATDC5 cells differentiated into brownish-black pigmented cells (Fig. 5C), although this occurred at very low frequency. It is possible that this differentiation was due to the contamination of other cells. To exclude this possibility, 20 randomly isolated subclones of ATDC5 were examined for their capacity to differentiate into both chondrocytes and pigmented cells. All those examined could differentiate into chondrocytes and at least 17 of them could also differentiate into pigmented cells. Therefore, ATDC5 cells

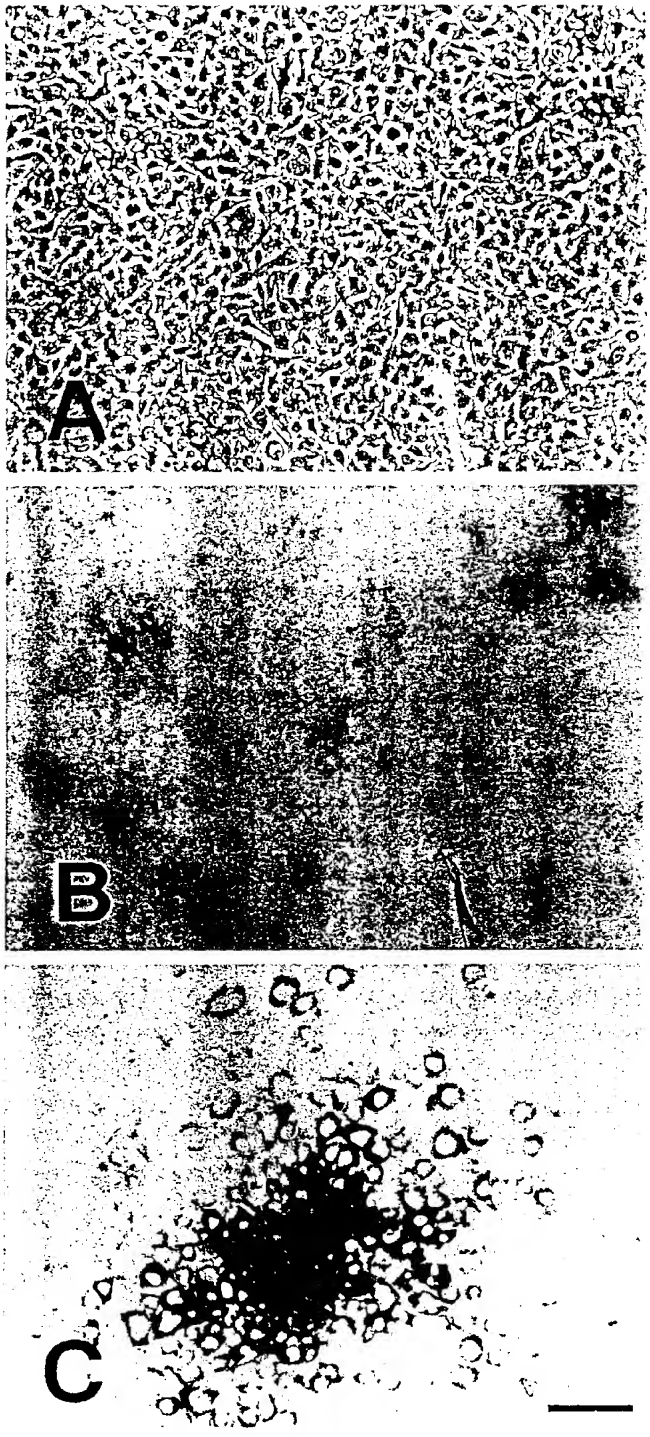

Fig. 5. (A) Phase-contrast micrograph of ATDC5 cell culture in a confluent phase where cartilage nodule-like cell aggregates had not yet formed; (B) the same field as (A) without phase-contrast. A few cells were already stained with anti-CSPG antibodies; (C) photomicrograph of pigmented cells differentiated from ATDC5. Bar, 50 μm.

were bipotent progenitor cells for chondrocytes and pigmented cells.

Discussion

We succeeded in isolating a cell line, ATDC5, from a differentiating culture of AT805 teratocarcinoma cells. ATDC5 cells easily formed cell aggregates when grown in a medium supplemented with insulin. The aggregates thus formed looked very similar to cartilage nodules which mesenchymal cells of chick embryo limb buds form when cultured at high density. In these aggregates ATDC5 cells showed a round morphology and the ability to synthesize both CSPG and type II collagen, which is characteristic of chondrocytes. Without the addition of insulin ATDC5 cells stopped growing when they became confluent, as observed with most normal cells. They neither formed cell aggregates nor were stained with Alcian blue. However, there were a few cells stained with anti-CSPG antibodies.

Insulin may play an important role in the growth and metabolism of developing cartilage (Silbermann, 1983). We have also shown here that insulin supported the proliferation of cells in a postconfluent phase and promoted the formation of cartilage nodule-like aggregates with chondrocyte-characteristic phenotype expressions. A few possibilities for the role of insulin can be pointed out: (1) insulin directly induces chondrogenic differentiation; (2) insulin induces cell proliferation even in a postconfluent phase and the resultant high cell density leads ATDC5 cells to differentiate into chondrocytes; (3) when insulin is present in the culture medium, unknown factors induce chondrogenic differentiation and only differentiated cells can continue to proliferate, even in a postconfluent phase.

With regard to the first possibility, if insulin directly induces the chondrogenic differentiation, ATDC5 cells should begin to differentiate when they are inoculated into medium supplemented with insulin. However, the characteristic chondrogenic differentiation of ATDC5 only occurred in a postconfluent phase; therefore, this possibility seems unlikely. The second possibility is supported by the fact that chondrogenic differentiation of chick limb bud cells in vitro is readily induced when the cells are seeded at high density (Solursh, 1983). The third possibility is also likely, because there were a few cells with the chondrogenic phenotype expression even in the cultures without the exogenous addition of insulin. The exact role of insulin in the chondrogenic differentiation of ATDC5 thus remains to be determined.

Since ATDC5 cells can also differentiate into pigmented cells, they are supposed to be at a prestage where cells have not yet determined to become chondrocytes. ATDC5 cells may be progenitor cells for chondroblasts. Darmon et al. (1984) reported that teratocarcinoma-derived mesenchymal cells could be converted into keratinocytes and astrocytes. Our results in this paper and theirs suggest that teratocarcinoma-derived mesenchymal cells (or embryonic mesenchymal cells) may have a greater capacity for differentiation than we have expected.

In comparison with other chondrogenic cell lines (C3H10T1/2 or RJC3.1), ATDC5 cells appear able to differentiate into chondrocytes at a very high frequency. This cell line will, therefore, be useful for studying the cellular and molecular mechanism of chondrogenic differentiation.

Acknowledgements

We thank Dr. H. Urushihara (Tsukuba University) for her critical reading of the manuscript and Dr. Y. Oike for his useful advice. This work was supported, in part, by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

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